EXHIBIT A

JOURNAL OF VIROLOGY, May 1992, p. 2934–2942
0022-538X/92/052934-09$02.00/0

Vol. 66, No. 5

Regulated Expression of Foreign Genes in Vaccinia Virus under the Control of Bacteriophage T7 RNA Polymerase and the *Escherichia coli lac* Repressor

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The gene encoding bacteriophage T7 RNA polymerase (*T7gene1*) was placed under the control of regulatory elements from the *Escherichia coli lac* operon to construct an inducible vaccinia virus expression system consisting entirely of prokaryotic transcriptional machinery. Regulated expression of T7 RNA polymerase was necessary to construct a stable recombinant vaccinia virus harboring a T7 promoter; otherwise, uncontrolled expression led to interference with endogenous virus replication. To this end, the gene encoding the repressor protein of the *lac* operon was fused to a viral early/late promoter so that it was expressed constitutively, and the *lac* operator was interposed between a viral major late promoter and *T7gene1*. Greater than 99% repression of T7 RNA polymerase, which was relieved approximately 80-fold in the presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), was obtained. An expression cassette containing a T7 promoter-controlled β-galactosidase reporter gene was recombined into a different region of the viral genome containing *T7gene1*. A stable, double recombinant virus was isolated and grown to a high titer. In the absence of inducer, β-galactosidase expression was substantially repressed. Addition of increasing amounts of IPTG induced expression of β-galactosidase to the point of suppression of viral replication. This hybrid vaccinia virus system (Vac/Op/T7) has potential applications for the efficient bioproduction of a wide variety of gene products.

Transcriptional and regulatory elements from viral or eukaryotic sources have been used extensively for the production and characterization of recombinant proteins in mammalian cells (16). These eukaryotic expression vectors typically carry genetic elements which confer drug resistance, the ability to replicate autonomously, and regulatory control to the target gene of interest. Although use of mammalian cells is essential in many instances for the synthesis of biologically active eukaryotic proteins, stable transformants are oftentimes difficult to construct and target proteins may be expressed at relatively low levels in comparison with their bacterial counterparts. Moreover, if tight regulation of transcription is required for the expression of specific protein sequences, regulated eukaryotic transcription systems such as the metallothionein promoter (2, 21) or the mouse mammary tumor virus promoter (17, 19) may not be suitable, since they are leaky under noninduced conditions and show rather modest levels of induction. On the other hand, promoters that are highly inducible, such as those responsive to glucocorticoid hormones (18), require the presence of hormone receptors in the cell, thereby restricting the range of cell types that can be used. As an alternative approach, a wide host range mammalian cell expression system incorporating desirable and highly regulatable prokaryotic transcriptional elements might have important advantages. For instance, the high catalytic activity, inducibility, and promoter specificity of several prokaryotic transcription systems have been well characterized. Thus, a eukaryotic expression system incorporating favorable transcriptional components from bacteria may offer a highly specific and efficient method for the biosynthesis of mammalian cell-derived proteins.

In previous reports (12, 15), we described a chimeric system that provides useful expression of recombinant proteins. This system, referred to as the hybrid Vac/T7 system, is based on coinfection of cultured cells with two recombinant vaccinia viruses: one recombinant virus provides constitutive expression of bacteriophage T7 RNA polymerase which transcribes a T7 promoter-controlled target gene in the second virus. Although the Vac/T7 system has been widely used, inherent limitations exist. While the requirement for two viruses may be advantageous under some circumstances (e.g., expression of toxic proteins), it adds to the expense and complicates the protocol. For instance, optimal levels of expression are dependent on cells being infected with similar amounts of each virus. In addition, differential rates of virus replication may limit the ability to establish a spreading infection at a low multiplicity of infection (MOI). Our attempts to simplify this two-virus vector system by making a single recombinant vaccinia virus containing both T7 RNA polymerase and T7 promoter elements have been unsuccessful, presumably because of interference with viral transcription and/or replication. If expression of T7 RNA polymerase could be negatively regulated and induced upon command, a single-virus expression system may be feasible (24).

Here we describe the development of a vaccinia virus expression system composed primarily of prokaryotic transcriptional elements. A preliminary description of this work has been reported previously (1). The genes that encode the T7 RNA polymerase and regulatory elements from the *Escherichia coli* lactose operon were inserted into the genome of vaccinia virus. A recombinant vaccinia virus that constitutively expresses the *lac* repressor was constructed,

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and the activity of T7 RNA polymerase was regulated by placing it under the control of a hybrid promoter in which the *lac* operator was inserted just downstream of a late vaccinia virus promoter. The activity of T7 RNA polymerase was regulated over an 80-fold range by the *lac* repressor, and this effect was reversible by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). A secondary gene cassette that contains the *E. coli* β-galactosidase (β-GAL) gene (*lacZ*) under control of a T7 promoter was inserted into the viral genome by using *E. coli* guanine-hypoxanthine phosphoribosyltransferase (*gpt*) as a dominant selectable marker. A stable vaccinia virus recombinant was isolated and grown to high titer. Induction of β-GAL expression could be determined to the point at which the virus ceased to replicate. In this communication, we demonstrate the powerful utility of developing chimeric expression systems in mammalian cells for the production of recombinant proteins.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. Mycophenolic acid (MPA) was obtained from Calbiochem Corp.; hypoxanthine and xanthine were obtained from Sigma Chemical Co. MPA and xanthine were dissolved in 0.1 N NaOH, and hypoxanthine was dissolved in water and sterile filtered; the solutions were stored frozen as 10-mg/ml stocks.

Virus and cells. Vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection, propagated in HeLa cells, and purified as reported previously (20). HeLa cells S3 were grown in Eagle minimal essential medium (EMEM) supplemented with 5% horse serum. Human thymidine kinase-negative (TK^-) 143 cells (26) were grown in EMEM containing 10% fetal bovine serum (FBS) and 25 μg of 5-bromodeoxyuridine (BUdR) per ml. CV-1 and BSC-1 cells were grown in Dulbecco modified Eagle medium containing 10% FBS.

Vector construction. The sequence immediately upstream of the translation start site from *T7gene1* was modified by M13 oligonucleotide-directed mutagenesis (11) to remove the potential upstream Shine-Dalgarno sequence (GAGG) as described by Stahl and Zinn (26a) and to insert unique restriction enzyme sites. To accomplish this construction, *T7gene1* was excised from pAR1173 (5) with *Bam*HI as a 2.6-kbp DNA fragment and inserted into the *Bam*HI site of the double-stranded, replicative form of mp19. The resulting vector was termed RFmpT7gene1. An oligonucleotide with complementary sequences flanking the Shine-Dalgarno motif, 5'-ATCGTGTTCATTTAAGATCTGAATTCGGATCCT CTAGAGT-3', was used to selectively remove the Shine-Dalgarno sequence while inserting *Bgl*II-*Eco*RI-*Bam*HI restriction sites (underlined). The *Bgl*II site abuts the 5' end of TAAATG where the thymidine (boldface) corresponds to T7 nucleotide 3168 (6) and the T7 RNA polymerase translation start site is underlined. The mutagenized, single-stranded template was converted to the replicative form by standard techniques, and the phage were screened by plaque assay with the ^{32}P-labeled mutagenizing oligonucleotide as a probe. Positive plaques were identified, and the mutagenesis was confirmed by restriction enzyme and DNA sequence analyses. Replicative form DNA was prepared from the recombinant phage termed RFmpTF7gene1-4 (designated pTF7gene1 in Fig. 2). A second modification of *T7gene1* was performed by M13 oligonucleotide-directed mutagenesis to engineer an *Eco*RI site coincident with the translation start site of the polymerase. An oligonucleotide, TF7-11, 5'-ATG TAAATCGAATTCATTTAAGGATCCTCTAGAGT-3', was synthesized to modify the native 5' sequence of *T7gene1*, . . .TAAATGAACACG. . ., to . . .TAAATGAATTC. . ., thus inserting an *Eco*RI site immediately downstream of the translation start site. This modification resulted in a conservative change in the third codon from a threonine to a serine without any apparent change in T7 RNA polymerase activity. Replicative-form DNA was prepared from the positive phage and was termed RFmpTF7gene1-Eco. Construction of recombinant plasmids pT7lacOI and pP11T7gene1 is described in the legend to Fig. 2.

To generate the transfer vector, pVacHAgpt, a 1.8-kbp *Sal*I-*Hin*dIII DNA fragment containing the vaccinia virus (strain WR) hemagglutinin (HA) locus was inserted into the *Eco*RI-*Hin*dIII sites of pUC19, in which the *Sal*I and *Eco*RI sites were made flush with Klenow polymerase. The resulting vector was termed pTFHA. A set of complementary oligonucleotides containing a multiple cloning site with the restriction enzyme sites *Sst*I, *Sma*I, *Xho*I, *Bam*HI, *Sal*I, and *Eco*RI (TF62, 5'-GGAGCTCCCCGGGCTCGGAGGGATC CGTCGACTACTGAATTC-3'; TF63, 5'-GAATTCCCAG GTCGTCGACGGATCCCTCGGAGCCCGGGGAGCTC-3') were annealed and inserted into the unique *Nru*I site in pTFHA. The multiple cloning site bisected the HA gene, and the recombinant plasmid, pVacHA, was isolated and purified. A 2.1-kbp *Eco*RI-*Sal*I DNA fragment containing the bacterial *gpt* gene encoding guanine-hypoxanthine phosphoribosyltransferase (25) regulated by the vaccinia virus P7.5 promoter was excised from pTK61-gptΔ*Bam*HI (9) and ligated to *Eco*RI-*Sal*I-cleaved pVacHA to create the vaccinia virus transfer vector, termed pVacHAgpt.

Recombinant virus isolation. Recombinant viruses vT7lacOI and vP11T7gene1 were prepared as described previously (20) by homologous recombination into the TK locus and selection for TK^- phenotypes in the presence of BUdR. To generate the recombinant virus vT7lacOIZ, CV-1 cells (3×10^6) were infected with 0.5 PFU of vT7lacOI per cell and then transfected with a calcium DNA precipitate consisting of 10 μg of supercoiled pPT7lacZ DNA, 1 μg of vT7lacOI DNA, and 14 μg of sheared calf thymus DNA. After 48 h, virus stocks were prepared by resuspending the infected cells in 1 ml of medium and freezing and thawing the mixture three times. Selection for recombinant viruses containing the bacterial *gpt* gene was accomplished by three successive rounds of plaque formation on BSC-1 cells in the presence of EMEM containing 2.5% FBS, 25 μg of MPA per ml (Calbiochem), 250 μg of xanthine per ml, and 15 μg of hypoxanthine per ml (9). The gpt^+ viruses obtained were then plated on BSC-1 cells without selection, and DNA from isolated viral plaques were analyzed by polymerase chain reaction and agarose gel electrophoresis for the inserted sequence.

β-GAL assay. Infected or transfected BSC-1 cells (10^6) were grown in 2 ml of EMEM (without phenol red) containing 2.5% FBS. The infected cells were harvested, 100 μl of $CHCl_3$ and 10 μl of 10% (wt/vol) sodium dodecyl sulfate were added, the cells were dispersed by vortexing, and the mixture was centrifuged to remove cellular debris. The supernatant was assayed for β-GAL activity by using *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (23). The reaction was performed in a 96-well microtiter plate, and the yellow color was quantitated by measuring A_{405} with a kinetic microplate reader (Molecular Devices).

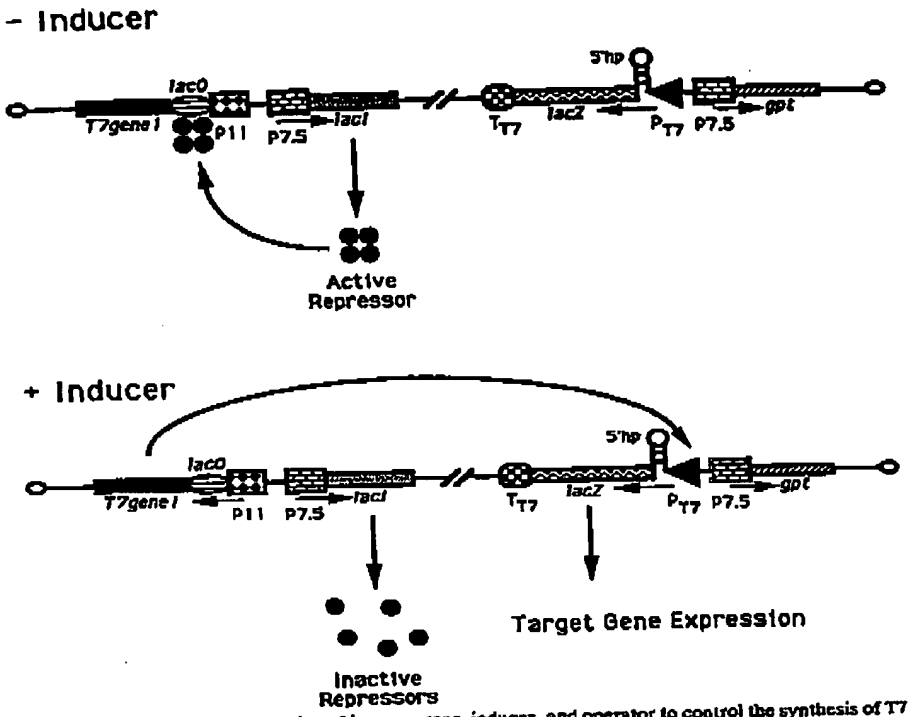

FIG. 1. Schematic representation of the interaction of *lac* repressor, inducer, and operator to control the synthesis of T7 RNA polymerase and subsequent induction of the target gene (*lacZ*). The *lac* repressor was synthesized at early and late times after infection by using the vaccinia P7.5 promoter. The active repressor binds to the *lac* operator (*lacO*) positioned between the vaccinia late promoter (P11) and the gene encoding T7 RNA polymerase (*T7gene1*). In the presence of inducer, the repressor is inactivated, and expression of T7 RNA polymerase is induced. T7 RNA polymerase initiates transcription from a T7 promoter (P_{T7}) and stops at the T7 terminator (T_{T7}). Chimeric T7-initiated transcripts are synthesized at high levels. A 5' stem-loop sequence (5'hp) stabilizes the transcripts from degradation. The dominant selectable marker, *gpt*, allows positive selection and retention of the target gene sequences inserted into the HA locus.

RESULTS

Construction of hybrid vaccinia virus promoters to control the expression of T7 RNA polymerase. A strategy was adopted to construct a recombinant vaccinia virus that was capable of regulated expression of T7 RNA polymerase and that contained a T7 promoter-controlled target gene (Fig. 1). We felt that regulation of T7 RNA polymerase expression was necessary, as previous attempts to construct a recombinant vaccinia virus containing both T7 RNA polymerase and a T7 promoter-controlled target gene were unsuccessful apparently because of the low viability of the double recombinant (12). Since small amounts of T7 RNA polymerase can direct most of the resources of an *E. coli* cell toward expression of a specific target gene, we reasoned that a similar event was occurring, thereby impeding the ability of the recombinant virus to replicate. Therefore, we considered the use of components from the *E. coli* lactose operon coding for the *lac* repressor (*lacI*) and its cognate operator sequence (*lacO*) to regulate the T7 RNA polymerase. Our objective was to construct a recombinant vaccinia virus that constitutively expressed *lacI* and that contained an appropriately placed *lac* operator sequence strategically positioned between a vaccinia virus late promoter (P11) and the coding sequence for T7 RNA polymerase (*T7gene1*). If stringent repression of T7 RNA polymerase was achieved, then stable insertion of a T7 promoter-controlled target gene could be maintained in a second location in the viral genome. Induction of T7 RNA polymerase expression would then result in significant T7 promoter-specific transcription initiation and target gene expression.

To regulate the expression of *T7gene1*, we first modified an expression vector that contained a *lacI* gene under control of the vaccinia virus early/late P7.5 promoter to allow the insertion of secondary gene cassettes (placIm, Fig. 2A). We chose the P7.5 promoter because it is transcriptionally active at early and late times after infection (29), thereby permitting constitutive *lac* repressor expression throughout the course of infection. Earlier studies demonstrated the utility of this approach in that a recombinant virus, vlacI, containing *lacI* stably integrated into the TK locus under the control of the P7.5 promoter, was used to successfully synthesize the functionally active repressor in infected cells. The amount of repressor expressed inhibited the expression of a vaccinia virus promoter-*lac* operator-*lacZ* gene fusion by up to 99.9% (13).

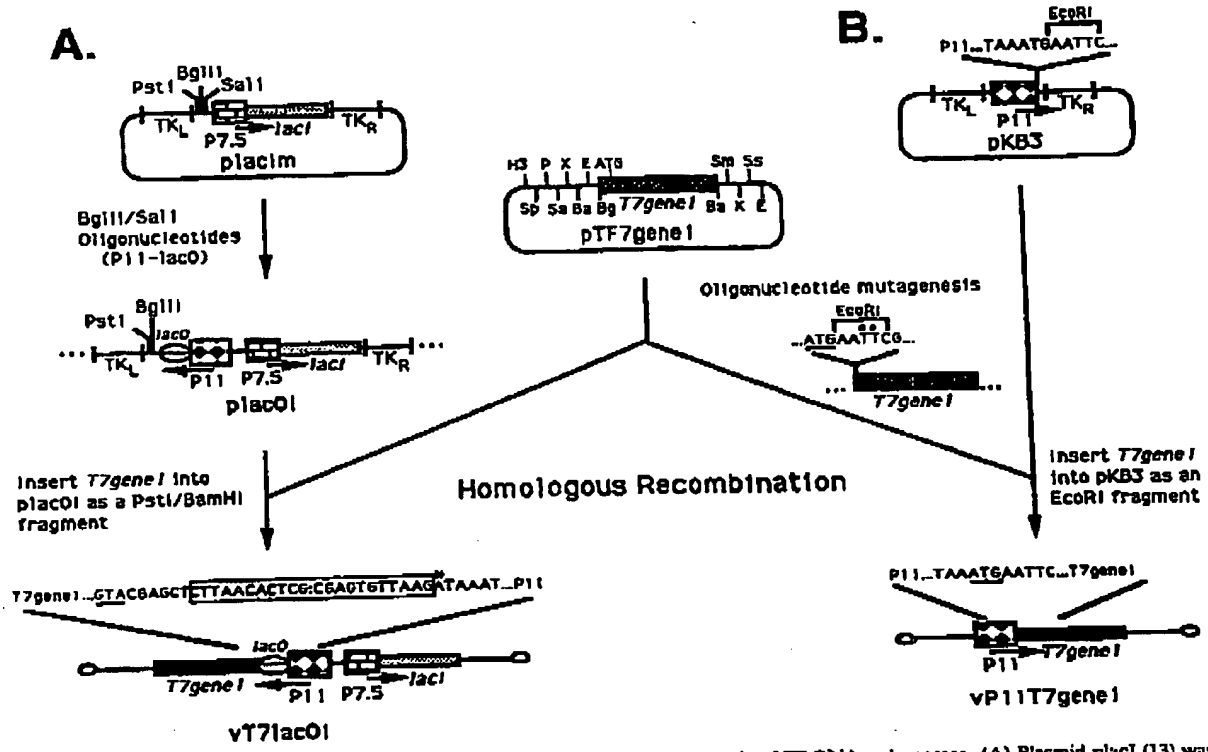

FIG. 2. Construction of recombinant vaccinia viruses which regulate the synthesis of T7 RNA polymerase. (A) Plasmid placI (13) was modified by inserting an 18-bp linker containing the restriction enzyme sites *BglII*, *XhoI*, and *PstI* into the *PstI* site, creating placIm. A 73-bp *BglII-SalI* compatible fragment, containing the vaccinia virus late promoter (P11) and *lacO* regulatory elements (P11-lacO), composed of the complementary and overlapping oligonucleotides BA1, BA5, BA6, and TF64, was inserted into the *BglII* and *SalI* sites of placIm to create placOI. The sequences of these oligonucleotides were 5'-TAGCATAGAAAAAAACAAAATGAAATTCTACTATATTTTTA-3' for BA1, 5'-CTATGCTATAAATAGAATTGTGAGCGCTCACAATTA-3' for BA5, 5'-GATCTAATTGTGAGCGCTCACAATTCTATTTA-3' for BA6, and 5'-TCGATAAAAATATAGTAGAATTTCATTTTGTTTTTTT-3' for TF64. Plasmid pT7lacOI was constructed by cleaving placOI with *PstI* and *BglII* and inserting a 2.7-kbp *PstI-BglII* fragment containing *T7gene1* from pTF7gene1 in which the Shine-Dalgarno fragment was removed. pT7lacOI was inserted into the vaccinia virus (strain WR) by homologous recombination (20), creating the recombinant virus vT7lacOI. The asterisk denotes the exchange of adenosine for guanosine in the native TAAATG sequence. The boxed area represents the 22-bp *lacO* sequence with dyad symmetry. (B) A 2.6-kbp *T7gene1 EcoRI* DNA fragment, in which an *EcoRI* site was engineered just downstream of the translation start site (see Materials and Methods), was inserted into the *EcoRI* site of the expression vector pKB3 (8) to form pP11T7gene1. This insertion vector was recombined into the TK locus of vaccinia virus (strain WR) by standard techniques, creating the recombinant virus vP11T7gene1. The underlined translation start site represents its native location in the context of the 11-kDa protein.

We then wished to place a synthetic *lacO* adjacent to a late promoter so that repressor binding would block transcription yet not severely disturb transcription in the absence of the repressor. The optimal site for placement of the synthetic *lacO* sequence, relative to a vaccinia virus late promoter, was previously described (13). Positioning of *lacO*, a 22-bp palindrome, immediately downstream of the highly conserved TAAAT motif of late promoters satisfied these criteria. The vaccinia virus late promoter for the gene encoding the 11-kDa structural protein (P11) was used in these studies, and the hybrid promoter was referred to as P11lacO. A set of four overlapping and complementary oligonucleotides encompassing P11lacO were annealed and inserted into the *BglII-SalI* sites of placIm to create placOI. The coding sequence for *T7gene1*, excised from pTF7gene1 in which the Shine-Dalgarno motif was removed and a unique *BglII* site was inserted, was juxtaposed immediately downstream of P11lacO, and the resulting plasmid was termed pT7lacOI (Fig. 2A). It was necessary to remove the Shine-Dalgarno sequence immediately upstream of the translation start site of *T7gene1* to permit fusion with the P11 promoter and subsequent propagation of the recombinant plasmid in *E. coli*. Since the P11 promoter is transcriptionally active in *E. coli*, presumably, the T7 RNA polymerase expressed is toxic to the cells (14a). In this configuration, the synthetic *lac* operator sequence was placed two bases downstream of the RNA start site of *T7gene1*. In addition, a recombinant plasmid, termed pP11T7gene1, in which *T7gene1* was fused to the naturally occurring translation start site of P11 which overlaps the TAAAT(G) sequence motif (Fig. 2B), was constructed. Although the presence of a guanosine immediately following TAAAT is not essential for late transcription,

substitution of adenosine has been shown to lower expression by approximately 25% (13). Therefore, both plasmids, pT7lacOI and pP11T7gene1, were constructed to compare the relative level of T7 RNA polymerase expressed from the natural and modified P11 promoters.

Regulation of T7 RNA polymerase expression. To determine whether genetic elements carried by pT7lacOI and pP11T7gene1 were functionally active, the recombinant plasmids were transfected separately into wild-type vaccinia virus-infected cells, and T7 RNA polymerase was assayed. In these transient assays, T7 RNA polymerase expression from pP11T7gene1 was unaffected in the absence or presence of the inducer, IPTG. However, substantial repression and subsequent induction in the presence of IPTG occurred in cells transfected with pT7lacOI (data not shown). Since T7 RNA polymerase appeared to be regulated, we proceeded to construct recombinant viruses containing these gene cassettes inserted into the TK gene by homologous recombination. TK$^-$ recombinant viruses, designated vT7lacOI and vP11T7gene1 (Fig. 2), were purified, and the correct insertion of the gene cassettes was confirmed by Southern blot hybridization.

The ability of the recombinant virus vT7lacOI to synthesize the functionally active *lac* repressor capable of binding to its cognate operator sequence in vitro was determined by mobility shift assay. A radioactively labeled synthetic operator containing the sequence GAATTGTGAGCGCTCAC AATTC and its complement were prepared as described previously (13). The 41-bp probe was incubated with dilutions of extracts made from cells infected with vlacI, vT7lacOI, or wild-type virus. Extracts prepared from both the *lacI*-containing viruses retarded the mobility of the probe (Fig. 3). The protein-DNA complex comigrated with the complex formed from the association of the authentic repressor binding to the probe. Since known amounts of purified repressor were used as a standard, we calculated by densitometry that approximately 2×10^7 repressor tetramers per cell are present after a 24-h infection. This corresponds to approximately 1,000 active tetramers for each replicated vaccinia virus genome, in agreement with previously reported values (13).

To determine the magnitude of repression of T7 RNA polymerase, and subsequent induction of expression, cells were infected with either vT7lacO1 or vP11T7gene1 in the presence or absence of IPTG. All comparisons were made relative to the amount of T7 RNA polymerase expressed from cells infected with a TK$^-$ recombinant virus, vP11T7gene1, containing the unmodified P11 promoter fused to *T7gene1*. Cells lysates were prepared and tested in vitro for T7 RNA polymerase activity. In the absence of IPTG, T7 RNA polymerase was repressed by >98% at an MOI of 0.1 and by more than 99% at higher MOIs (Table 1). With the addition of IPTG, the level of T7 RNA polymerase activity was 58% of maximum levels obtained in the absence of the repressor, resulting in more than 80-fold induction. By contrast, IPTG had negligible effect on T7 RNA polymerase expression in cells infected with vP11T7gene1.

Construction of a single recombinant vaccinia virus containing *T7gene1* and T7 promoter elements. To test the feasibility of constructing an inducible, single-virus system, we sought to incorporate a T7 promoter-controlled target gene into the recombinant virus, vT7lacOI. A new transfer vector for insertion into the HA locus was constructed, as the *T7gene1* and *lacI* elements were already inserted into the TK locus. Previous studies have shown that foreign gene insertion into the vaccinia virus HA locus does not interfere with the ability of the HA recombinant virus to replicate in vitro (10). To overcome a potential problem of selection and retention of target gene sequences in the HA locus, the *E. coli gpt* gene under control of the vaccinia P7.5 promoter was used as a dominant selectable marker (9). Since mammalian cells and vaccinia virus cannot use xanthine for GMP synthesis when de novo purine synthesis is blocked, i.e., in the presence of MPA, vaccinia virus expression of *gpt* can overcome this block and allow replication to continue. The *E. coli lacZ* gene encoding β-GAL was chosen as a reporter because the assay is quantitative and there is no detectable background of β-GAL in mammalian cells (15). Moreover, we anticipated that induction of blue plaque formation, in the presence of inducer and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), may also be used for screening. Therefore, an insertion vector was constructed, termed pPT7lacZ, containing these genetic elements with *lacZ* fused to a T7 promoter (Fig. 4). Cells infected with vT7lacOI (Fig. 2A) were transfected with pPT7lacZ, and 2 days later, cell lysates were prepared and plaqued on BSC-1 monolayers in *gpt* selection medium. A stable recombinant virus, designated vT7lacOIZ, was isolated, plaque purified three times, and grown to a high titer.

To test the inducibility of this system, BSC-1 cell monolayers were infected with 50 PFU of vT7lacOIZ with or without IPTG, and plaques were stained for β-GAL activity in the presence of X-Gal. As shown at the bottom of Fig. 4, in the absence of IPTG, plaques displayed a faint staining

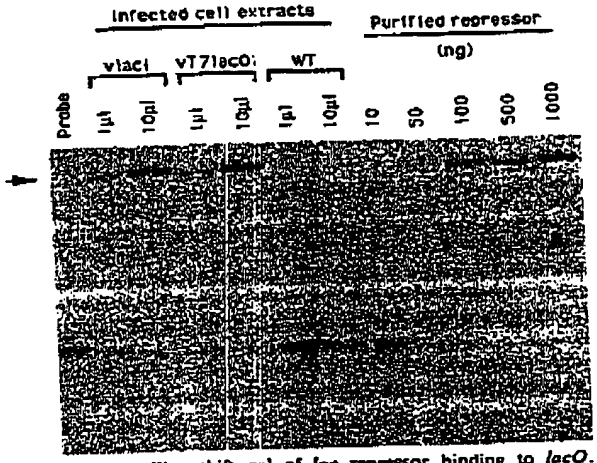

FIG. 3. Mobility shift gel of *lac* repressor binding to *lacO*. Approximately 2×10^7 CV-1 cells were infected with vlacI, vT7lacOI, or wild-type vaccinia virus at an MOI of 10. Cells were harvested 24 h after infection, and cytoplasmic fractions were prepared as described previously (13). Extracts (1 and 10 μl) were mixed with ^{32}P-labeled, double-stranded oligonucleotide probe and 50 mM poly(dI-dC). The probe was composed of the oligonucleotides PE7 and PE8 annealed together, and the nonoverlapping nucleotides were filled in with [α-^{32}P]dCTP. The sequences of these oligonucleotides were 5'-CTATGCTAGAATTGTGAGCGCTCAC AATTCTAAATAC-3' for PE7 and 5'-TCGAGTATTTAGAATTG TGAGCGCTCACAATTC-3' for PE8. In addition, various amounts of purified *E. coli lac* repressor protein were mixed with probe and poly(dIdC) to serve as a positive control. The samples were separated by 8% polyacrylamide gel electrophoresis and exposed to X-ray film. Arrowhead, *lac* repressor bound to *lacO* probe.

TABLE 1. Regulation of T7 RNA polymerase expression from vT7lacOI-infected cells[a]

Virus	Promoter junction sequence	MOI	cpm of ^{32}P incorporated (% expression)[b]		Induction (fold)
			Without IPTG	With IPTG	
vT7lacOI	P11-TAAATA-Op-T7gene1	10	1,371 (0.72)	111,811 (58.7)	81.6
		1	1,600 (0.84)	88,192 (46.3)	55.1
		0.1	2,671 (1.35)	44,572 (23.4)	16.7
vP11T7gene1	P11-TAAATG-T7gene1	10	190,480 (100)	189,527 (99.5)	None

[a] BSC-1 cell monolayers were infected with vT7lacOI or vP11T7gene1, with or without IPTG, at the indicated MOI. Cell lysates were prepared 24 h after infection. T7 RNA polymerase activity in cell lysates was assayed by using a DNA template containing a T7 promoter as described previously (15).
[b] Expression values are given as the amount (cpm) of ^{32}P-labeled ribonucleotide incorporated in a standard reaction as well as the amount (%) relative to the maximum activity obtained.

pattern, presumably because of the very low, yet detectable, level of T7 RNA polymerase expressed (see Table 1). However, plaques formed in the presence of 10 μM IPTG stained dark blue when IPTG was added at the time of infection, demonstrating the inducibility of this system.

Effect of IPTG on virus replication and β-GAL expression. Initial experiments (1) indicated that high concentrations of IPTG specifically prevented plaque formation by vT7lacOIZ. These observations led us to investigate the effect of IPTG on the growth of vT7lacOIZ. Single-step growth curves were established for the recombinant virus vT7lacOIZ and its parent lacking the PT7lacZ cassette, vT7lacOI. The addition of 1 mM IPTG at the time of infection had no effect on the ability of vT7lacOI to replicate over a 24-h period (data not shown), whereas it completely abrogated replication of vT7lacOIZ (Fig. 5A), presumably because of interference of endogenous viral transcription and/or replication. In fact, replication of vT7lacOIZ was arrested when 1 mM IPTG was added at early or late times after infection, indicating a pleiotropic adverse effect on replication. We then determined the concentration of IPTG at which virus replication became compromised (Fig. 5B). As little as 25 μM IPTG began to inhibit virus replication, and 50 μM IPTG completely abolished formation of infectious virus.

To determine the concentration of IPTG that would result in minimum inhibition of virus replication yet yield the highest level of β-GAL expression, several doses of IPTG were tested for induction. β-GAL activity present in lysates of BSC-1 cells infected 24 h earlier with 10 PFU of vT7lacOIZ per cell by using a range of 1 to 100 μM IPTG was measured quantitatively as described in Materials and Methods. A maximum level of expression was obtained by using 15 μM IPTG, which was found to have a small inhibitory effect on vT7lacOIZ replication. At this concentration of IPTG, a 5- to 10-fold induction of β-GAL activity was observed. These results demonstrate that a high degree of T7 RNA polymerase repression was achieved. This repression could be reversed, resulting in a considerable induction of β-GAL activity at low IPTG concentrations.

We next wished to determine whether maximum β-GAL expression from cells infected with vT7lacOIZ could be achieved through continuous induction at the time of infection or a burst of induction at the late times after infection. These studies, in which the single inducible virus is referred to as the Vac/Op/T7 system, were performed in comparison to the previously described hybrid Vac/T7 coinfection system (12). By using optimal conditions for each system, cells were infected with vT7lacOIZ (Vac/Op/T7) or coinfected with recombinant vaccinia viruses vTF7-3 and vTF7LZ-1 (Vac/T7) in the absence or presence of either 15 μM or 1 mM IPTG added early (2 h) or late (12 h) after infection. (Recombinant virus vTF7-3 expresses T7 RNA polymerase and vTF7LZ-1 contains the PT7lacZ cassette.) Cell extracts were prepared 24 h after infection and assayed for β-GAL expression by colorimetric assay. As shown in Table 2, the highest β-GAL activity was obtained when cells were infected at an MOI of 10 in the presence of 15 μM IPTG added at 2 h postinfection (100%). This activity is approximately twofold greater than that obtained for the coinfection system. Similar results were obtained when cells were infected with vT7lacOIZ or coinfected by using the Vac/T7 system in the presence or absence of IPTG and β-GAL protein was measured by immunoblot analysis (1). On the basis of this analysis, with purified β-GAL as a standard, we estimated that approximately 5 μg of β-GAL per 10^6 cells was made 24 h after infection by the Vac/Op/T7 system.

DISCUSSION

We have described a unique mammalian cell expression system in which prokaryotic transcriptional and regulatory elements were used to control the expression of a foreign gene carried by an animal virus host vector. We selected bacteriophage T7 RNA polymerase for its highly specific and catalytic properties and regulatory elements from the *E. coli* lactose operon for their ability to block transcription over several orders of magnitude. Vaccinia virus was chosen as a desirable vector system because it has a cytoplasmic mode of replication and it encodes mRNA modification enzymes which appear necessary for the translation of T7-specific transcripts (3, 7). In addition, vaccinia virus can replicate in a wide host range of cell types and amplifies its genome from 10,000 to 20,000 copies per cell, thereby increasing the copy number of the target gene template.

Initial attempts were made to incorporate a T7 promoter into the genome of a vaccinia virus recombinant that constitutively expressed T7 RNA polymerase. These attempts were unsuccessful, presumably because of the high catalytic properties of the polymerase, resulting in the interference with viral transcription and/or replication. Similar observations have been reported for *E. coli* with which even basal T7 RNA polymerase expression present in an uninduced cell can prevent, in some cases, target genes from being established in the same cell. In fact, the use of T7 lysozyme to inhibit such basal transcription was necessary to overcome this problem (4). These observations support the finding that relatively small amounts of T7 RNA polymerase can direct most of the resources of an *E. coli* cell toward expression of a target gene (27, 28). Furthermore, cells infected with a vaccinia virus recombinant expressing T7 RNA polymerase can direct 30% of total cellular RNA to be initiated from a T7 promoter (14). Therefore, transfer of the *E. coli lac* operator-

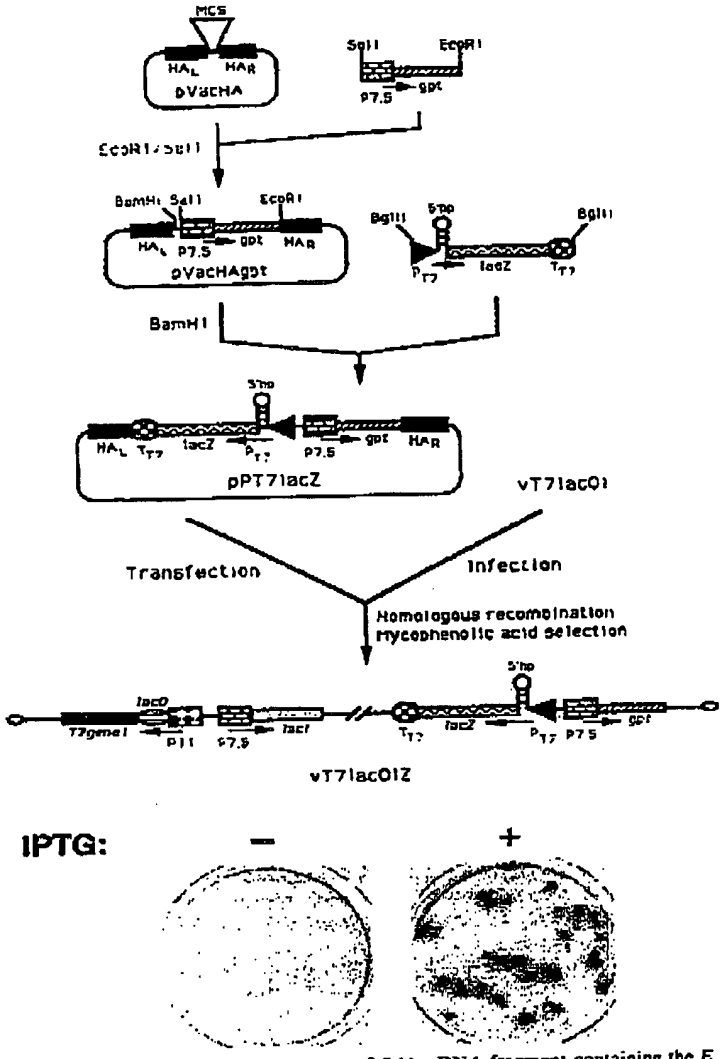

FIG. 4. Construction of a single, inducible recombinant virus. A 3.3-kbp DNA fragment containing the *E. coli lacZ* gene flanked by T7 promoter (P_{T7}) and transcription terminator (T_{T7}) elements was cleaved with *Bgl*II from pTF7LZ-1 (12) and inserted into the *Bam*HI site of pVacHAgpt. The resulting insertion vector, pPT7lacZ, was then recombined into the HA locus of recombinant vaccinia virus vT7lacO1 by homologous recombination. By using MPA selection (see Materials and Methods), a recombinant virus termed vT7lacO1Z was isolated and grown to a high titer. Induction of β-GAL expression was detected by plaque assay. Confluent BSC-1 cell monolayers were infected with 50 PFU of vT7lacO1Z per well in the absence (–) or presence (+) of 10 μM IPTG added at the time of infection. After a 2-day incubation, the cell monolayers were stained with X-Gal for 8 h.

repressor system to regulate the expression of T7 RNA polymerase was essential for stable incorporation of a T7 promoter-controlled reporter gene.

The stringency of repression of T7 RNA polymerase was tested by using a hybrid promoter in which *lacO* was positioned immediately downstream of vaccinia virus late promoter P11. The optimal positioning of the *lacO* sequences was previously determined by using β-GAL as a reporter gene. Using the same promoter-operator configuration, we found that up to 99.9% repression of β-GAL expression was obtained (13). Similarly, in this study, T7 RNA polymerase was repressed greater than 99% at higher MOIs. Addition of IPTG, however, induced expression to values at least 50% of maximum, resulting in an overall

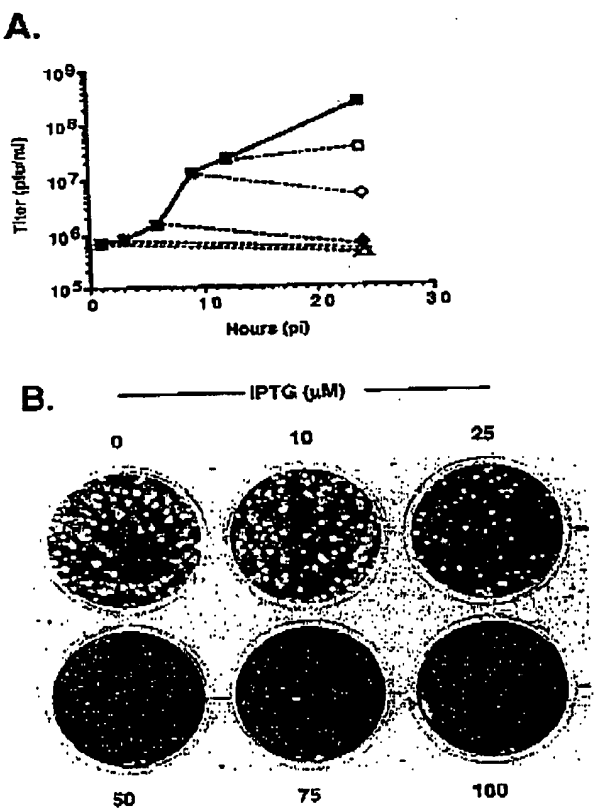

FIG. 5. Effects of IPTG induction on vT7lacOIZ replication. (A) BSC-1 cell monolayers were infected with vT7lacOIZ at an MOI of 1 PFU per cell. After 1 h, the cells were washed with EMEM containing 2.5% FBS and then overlaid with the same medium. vT7lacOIZ-infected cells (■) were incubated in the absence or presence of 1 mM IPTG added at 1 (△), 3 (+), 6 (◆), 9 (◇), or 12 (□) h postinfection (pi). Cell lysates were prepared at the indicated time points, including 24 h after infection, and titers for virus were determined by plaque assay. (B) Confluent BSC-1 cell monolayers were infected with 100 PFU of vT7lacOIZ per well in the absence or presence of the indicated concentrations of IPTG. After a 2-day incubation, the cell monolayers were stained with a solution of 0.1% (wt/vol) crystal violet in 20% ethanol.

80-fold induction in polymerase activity. Interestingly, when the reporter gene was placed under the control of a T7 promoter and tested in this system, only a sixfold induction was observed at the higher MOI (Table 2, MOI = 10). This significant basal level of uninduced β-GAL activity probably results from the barely detectable amount of T7 RNA polymerase activity formed in the absence of inducer. If necessary, a still higher degree of repression may be obtained by increasing the amount of repressor expressed and/or by using multiple copies of the operator. Since there was an approximately 1,000-fold excess of active repressor tetramer molecules present for each replicated vaccinia virus genome 24 h after infection, the latter approach of using multiple operators seems reasonable. In fact, insertion of a

TABLE 2. Comparison of β-GAL expression from the Vac/Op/T7 system versus the Vac/T7 coinfection system[a]

IPTG (mM)	Time (h)	β-GAL expression (%)[b]			
		Vac/Op/T7 at MOI of:		Vac/T7 at MOI of:	
		1	10	1	10
0		8.0	16.5	24.6	40.7
0.015	2	43.0	100.0	27.8	47.6
0.015	12	39.8	74.7	NT	NT
1.0	2	17.1	52.4	26.3	42.8
1.0	12	44.2	80.6	NT	NT

[a] BSC-1 cell monolayers were infected with vT7lacOIZ (Vac/Op/T7) or coinfected with vTF7-3 and vTF7LZ-1 (Vac/T7), with or without IPTG, at the indicated MOIs. IPTG was added either 2 or 12 h after infection. Cell lysates were prepared 24 h postinfection and assayed for β-GAL activity as described previously (15).

[b] β-GAL expression values (%) are relative to the maximum activity obtained. NT, not tested.

lac operator just downstream of a T7 promoter strongly represses transcription in *E. coli*, yet the usual high levels of expression are obtained after induction (4).

An interesting observation demonstrating the potency of the Vac/Op/T7 system was the ability to titrate the repression of virus replication by using increasing concentrations of IPTG. This effect could be due to the burden of RNA overproduction and/or read-through transcription into distally located viral transcription units, causing disruption of normal gene function. With regard to the latter, the T7 late terminator, TΦ, has been shown to terminate T7 RNA polymerase procession 80 to 90% of the time either in vitro or in vivo (5, 22). Although Northern (RNA) blot analysis of T7-initiated transcripts from the hybrid Vac/T7 system indicated that the T7 termination signal was effectively used, S1 nuclease analysis suggested that read-through transcription also occurred (14). Whether this amount of read-through impedes virus replication needs to be determined. Since TΦ is structurally similar to the rho-independent class of *E. coli* terminators, use of strong ribosomal terminators, such as rrnBT1, may be one approach for improving the stringency of transcription termination.

The general utility of this single virus Vac/Op/T7 system was demonstrated for the production of proteins at levels higher than those achieved by using the Vac/T7 coinfection system. Moreover, as optimal gene expression by using the Vac/T7 system is dependent on cells being coinfected with equal MOIs of two recombinant viruses, the single-virus Vac/Op/T7 system could be used at a low MOI to establish a spreading infection. Selection of viral mutants with a more persistent or prolonged infection phenotype in vitro, with reduced ability to replicate in vivo, may offer significant advantages and is under investigation. For these reasons, the Vac/Op/T7 system may be more economical, easier to use, and less subject to variation than a coinfection system for large-scale protein production.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant 1R43-GM43638 from the National Institutes of Health.

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